

Original Research Article

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## Production of Alkaline Keratinolytic Protease by *Bacillus* sp. B13 from Feather Waste

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### ABSTRACT

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Alkaline keratinolytic protease producing *Bacillus* species was screened out from soil sample and characterized by studying morphological, cultural and biochemical properties. It was identified by using Burgey's manual of determinative bacteriology, identification flow charts as *Bacillus alcalophilus*. The isolate was employed for the production of alkaline protease by using production medium consisting of glucose and casein as carbon and nitrogen sources at pH 8.5 (Rao and Narasu, 2007). The isolate was also checked for its protease production by using feathers as sole source of carbon and nitrogen and produced significant amount of alkaline keratinolytic protease from feathers. The production was optimized for different criteria like, temperature, pH of production medium, substrate concentration (feather), incubation period of production, inoculum size. The alkaline protease produced from feather waste was also checked for its detergent compatibility and washing performance. It was found most compatible with detergent Wheel retaining 99.46% activity and with remarkable washing performance hence the enzyme can be used as detergent additive.

### Introduction

Keratin-containing materials are insoluble and resistant to degradation by the common proteolytic enzymes. Keratinous wastes represent a source of valuable proteins and amino acids. Keratinolytic enzymes have potential roles in biotechnological processes involving keratin containing wastes from poultry and leather industries. Keratinolytic proteases have different application where keratins should be hydrolysed such as the leather and detergent industries, textiles waste bioconversion, medicine, cosmetics and many

more novel outstanding applications. Several different strains of *Bacillus*, including *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. halodurans* or *B. pseudofirmus* are described to possess the ability of keratin biodegradation. *Bacillus* spp are known for production of wide variety of hydrolytic enzymes. So in this study we concentrated our study for isolating and characterizing one such *Bacillus* which degrades feather waste and produces alkaline protease from a cheaper raw material.

## **Materials and Methods**

### **Alkaline protease producing microorganisms: source and screening**

Various samples of soil and water were collected from different regions of Delhousie of Himachal Pradesh, Manikarnam near Manali (HP), J and J college of Science, Nadiad, Gujarat, samples were aseptically collected from top soil surface.

One gram of soil sample was diluted in 10 ml of sterile distilled water to make the sample and 1 ml of water sample of each site were serially diluted using sterile distilled water, stirred thoroughly and 100µl aliquots were spread on skim milk agar medium of pH: 8.5 and incubated at 37 °C for 2-3 days to allow the colonies to grow. The well isolated colonies were marked and colony characters and morphological characters were noted at the interval of 24 h, diameter of zone of clearance of casein was also measured which provided a measure of their Proteolytic activity.

### **Measure of proteolytic activity on solid media**

Fresh culture isolates producing zone of casein hydrolysis were taken and small drop was put in the middle of skim milk agar plate and incubated at 37°C for 5-6 days and at interval of 24 h., zone of casein hydrolysis and diameter of growth were measured and relative enzyme activity (REA) was calculated (Jain *et al.*, 2009).

(REA =Diameter of zone of casein hydrolysis/ Diameter of colony in mm.)

Based on REA, organisms were categorized into three groups showing excellent (REA>5), good (REA>2.0 to, 5.0) and poor (REA<2) producer of protease.

### **Study of cultural, morphological and biochemical characteristics**

The isolates showing zone of casein hydrolysis on milk agar plates, were studied for their colony characteristics, morphological and biochemical characteristics. Their morphological characteristics were studied by performing Gram's staining (Bergey *et al.*, 1994). Size of the isolate was measured by micrometry and endospore staining was done by Dornor's method. Their colony characters taken in consideration were, colony size, shape, elevation, margins, opacity, pigmentation, reverse side pigment, pigment solubility, texture etc. The biochemical tests carried out for the isolate were: indole production test, methyl red test, Voges proskauer test, citrate utilization test, nitrate reduction test, ammonia production test, catalase test, urea utilization test, gelatin hydrolysis test, hydrolysis of starch, H<sub>2</sub>S production test, dehydrogenase test. Growth pattern in broth, Carbohydrate utilization test etc. The classical method described in the identification key by Nonomura (1974) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974) was useful in the identification of Bacilli.

### **Study of growth characteristics**

#### **Measure of growth curve of isolate B13**

0.1 ml culture was inoculated in nutrient broth, mixed well and immediately at 0 h O.D was checked at 670nm. Then it was incubated at 37 °C and at the interval of every 2h, O.D. was measured until the growth declined. The growth curve was plotted with optical density against time.

#### **Effect of pH on growth of isolate B13**

0.1 ml culture was inoculated in nutrient broth with different pH mixed well and immediately 0 h O.D checked at 670nm. Then

it was incubated at 37°C and at the 24h, O.D. was measured until the growth.

### **Effect of osmotic pressure on growth of B13**

0.1 ml culture was inoculated in nutrient broth with different salt concentration (gm%), mixed well and immediately at 0 h O.D was checked at 670nm. Then it was incubated at 37°C and at the 24h, O.D. was measured.

### **Measure of alkaline protease production in routine medium and in medium with feathers as sole source of carbon and nitrogen**

To compare the caseinolytic activity of enzyme produced by the isolates, the isolates were inoculated in production medium consisting of glucose 150mg, K<sub>2</sub>HPO<sub>4</sub> 20mg, KH<sub>2</sub>PO<sub>4</sub> 20mg, MgSO<sub>4</sub> 10mg, CaCl<sub>2</sub> 10mg, casein 200mg, NaNO<sub>3</sub> 100mg, 100ml distilled water, pH-8.5 (Rao and Narasu, 2007) and put on an environmental shaker at 100 rpm at 37°C for 72 h and checked for enzyme activity at interval of 24h.

The supernatant was collected after centrifugation at 10,000 rpm for 15 minutes and used as crude enzyme source. Keratinolytic activity in the supernatant was determined by using spectrophotometer method, given by Anson - Hagihara (1958) with minor modifications. Same exercise was repeated by inoculating the culture inoculums in the production medium containing feathers as sole source of carbon and nitrogen (Feathers 0.5 gm, K<sub>2</sub>HPO<sub>4</sub> 20mg, KH<sub>2</sub>PO<sub>4</sub> 20mg, MgSO<sub>4</sub> 10mg, CaCl<sub>2</sub> 10mg, NaNO<sub>3</sub> 100mg, 100ml distilled water, pH-8.5). Activity of enzyme was measured in terms of unit. (µg/ml/min) One unit of enzyme is defined as the quantity of enzyme required to release 1µg of tyrosine per minute, under the standard assay conditions (Hameed *et al.*, 1999).

## **Optimization of production parameters**

### **Incubation period**

B13 was inoculated in 250ml Erlenmeyer flasks containing 100 ml of production medium, consisting of feathers as sole source of carbon and nitrogen and put on an environmental shaker at 100 rpm at 37°C for 72h and checked for enzyme activity at interval of 24h. The supernatant was collected after centrifugation at 10,000 rpm for 15 minutes and used as crude enzyme source.

### **Incubation temperature**

Effect of temperature on the production of extracellular protease production was analyzed by inoculating the isolate in various 250ml Erlenmeyer flasks containing 100 ml of production medium and then incubated at different temperatures (25, 37, 45°C) on environmental shaker at 100 rpm for 48h. After incubation period, from each flask, protease production was analyzed for optimum temperature for maximum protease production by the isolate.

### **Initial pH of the medium**

Effect of Initial pH of the production medium on production of extracellular protease was studied by assaying the enzyme after 48h of incubation at 37°C by adjusting the initial pH of the production medium to different pH values ranging from 5.0 to 10 using appropriate buffers. Tris HCl buffer (pH 6.0 - 8.0), Glycine NaOH buffer (pH 8.0-11).

### **Feather concentration (Substrate)**

The effect of Feather concentration on keratinolytic protease production by *B13* was carried out by inoculating 250ml Erlenmeyer flask containing 100 ml of production medium with the different concentration of the feather 0.2%, 0.3% 0.4%, 0.5%, 0.6% and

1% gm% and incubating for 48h at 37°C, on an environmental shaker at 100rpm. After incubation period, protease production was checked and results were analyzed for optimum substrate concentration for maximum protease production by the isolate.

### **Inoculum size**

The effect of Inoculum size of the culture *B13* was carried out by inoculating 250ml Erlenmeyer flask containing 100 ml of production medium with the different volumes of the inoculum 1.0 ml, 2.0 ml, 3.0 ml, 4.0 ml and 5.0 ml (OD 0.75 at 670nm) of isolate for 48h at 37°C, on an environmental shaker at 100rpm. After incubation period, protease production was checked in terms of protease activity and results were analyzed for optimum inoculum size of the culture for maximum protease production by the isolate.

### **Detergent compatibility and washing performance of keratinolytic alkaline protease**

The protease solution was pre incubated with different commercially available detergents like Tide, Rin, Ariel and wheel etc (Kumar and Bhalla, 2004). Detergent solutions were prepared in 1 gm% and 0.3 ml of detergent solution was mixed with 1.0 ml of enzyme solution to make final concentration of detergent to 7.2 mg/ml. the caseinolytic activity was determined at 37°C using Glycine NaOH buffer (pH 9.0) by Anson and Hagihara (1958) method. The relative activity was calculated with respect to the control without treatment, with any detergents.

Application of protease from isolate B13 as a detergent additive was studied by checking washing performance of protease on white cotton cloth pieces (10X 10 cm) stained with chocolate, as par (Adinarayana *et al.*, 2003; Kumar and Bhalla, 2004). The following sets were prepared for the study.

(A)Trey with 100 ml D/W + cloth stained with chocolate.

(B)Trey with 100 ml D/W + cloth stained with chocolate +2ml crude enzyme.

(C)Trey with 100 ml D/W + cloth stained with chocolate +2ml crude enzyme + 2ml 1% detergent.

(D)Trey with 100 ml D/W + cloth stained with chocolate + 2ml 1% detergent.

The trays were incubated at 37°C for 30 min. After incubation, cloths pieces were taken out, rinsed with water and dried. Visual examination of various pieces exhibited the effect if enzyme in removal of stains.

## **Results and Discussion**

### **Screening of protease producing microorganisms**

Screening of alkaline protease producing bacteria from various sources was carried out using alkaline skim milk agar medium. Out of various isolates three most potent isolates were collected showing zone of casein hydrolysis surrounding their colonies. All were alkaliphilic and having diverse morphological characters. The figure 1 and table 1 is representing the growth of these isolates on skim milk agar plates.

All three isolates were studied for casein hydrolysis zone size, colony characteristics and morphological characters (Table 1).

### **Comparative REA (relative enzyme activity) of isolates**

On the basis of morphology and cultural characteristics, it was confirmed that the isolates A1, and M9 were protease producing Actinomycetes and isolates B13 were Bacillus and all were producing good amount of alkaline protease on solid media. This was confirmed by performing REA (Figure 2 and

3). Highest REA 3.57 was observed for Bacillus strain B13. Similar reports were made by Richa Jain (2009) for various *Streptomyces* species by the similar method (Figure 2).

### **Selection of potent protease producing isolates**

Most of the isolates were having REA more than 2.0. So we counter checked them for their protease production capacity in production medium suggested by Rao and Narasu, 2007. When production profile of all the isolates was compared, Bacillus B13 was found producing maximum amount of protease within 48 h (140 units/ml) (Figure 3). B13 produced highest keratinolytic protease among three isolates (117.5 units/ml within 48h) using feathers as sole source of carbon and nitrogen. Though B13 produced protease using casein and glucose as carbon and nitrogen source (140.62 units/ml) higher than that of by using feathers as sole source of carbon and nitrogen (117.5 units/ml), as feathers are cheaper raw material, we selected feathers as substrate for enzyme production

### **Identification of potent protease producing isolate B13**

As we have decided to work with Bacillus, most explored bacteria for enzyme production. It was producing enzyme faster within 48h in larger quantities using feathers only hence, we selected B13 for further studies. For its identification for we relied upon: Cultural and morphological characteristics, Biochemical characters and Growth parameter.

### **Morphology, Cultural characteristics and Biochemical characteristics of B13**

The cultural characteristics, morphological characteristics and spore nature of the isolate B13 are presented in Table.2 and photographs

of the growth characteristics and gram staining are presented in figures 1, 5a and b. Results of biochemical tests carried out for isolate B13 are presented in Table:3.

### **Growth parameters**

Various growth parameters of isolate were studied like, Growth curve, of isolate B13, effect of environmental pH and osmotic pressure on the growth. Growth curve (Figure 6) indicated that isolate grows best within 20h and produces protease in its late stationary phase of life cycle and isolate B13 grows best at pH 11 indicating that isolate is alkaliphilic (Figure 7). When effect of osmotic Pressure on growth of isolate B13 was studied, it was found that isolate B13 can grow in the range of 0.5gm% to 6.5gm% of NaCl (Figure 8).

### **Protease production and optimization of certain parameters**

#### **The protease production profile of B13 to determine incubation period of fermentation**

The study of enzyme production is presented in figure 9. In which it was found that incubation period for best production was 48h with maximum protease activity (117.5 units/ml). So throughout the study we considered 48h as incubation period for the fermentation.

Similar kinds of results were also reported for *B. subtilis* AKRS3 Krishnan Ravishankar *et al.*, 2012. Study of growth patterns indicated that maximum growth of isolate B13 (Figure: 9) was found at 20 h while the enzyme production was optimum at 48 h indicating that protease was produced maximally in late stationary phase of growth. Result was quite similar to that of reports for *Streptomyces clavuligerus* (Keila *et al.*, 2001).

### **Effect of temperature on protease production by isolate B13**

The growth and enzyme production are greatly influenced by incubation temperature. It was found that 37°C was the most favorable temperature for protease production by the isolate B13 (Figure 10). Similar reports were recorded for *B.subtilis* by Amira Hassan AL-Abdalall and Eida marshid Al-khaldi (2016); and for *B.cereus* by Jeevana Lakshmi, kumari chitturi and lakshmi (2013).

### **Effect of Initial pH of medium on protease production by isolate B13**

Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH.

The results showed (Figure 11) that the enzyme production was maximum at pH 10.(106.5units/ml) Our result matches with the reports like, growth and protease production were maximum at pH 10 for *Bacillus clausii* by Denizci *et al.*, (2003) and *Bacillus sp.* JB 99 by Pushapalata Kainoor and Naik (2010)

### **Determining optimum feather concentration of production medium for protease production by isolate B13**

Optimum feather concentration of production medium for keratinolytic protease production by isolate B13 was found 0.5% (82.5units/ml). Similar results were noticed for organism *Thermoactinomyces sp.* RM4 by Amit Verma *et al.*, (2016).

### **Effect of inoculum size on protease production by isolate B13**

Inoculum size also affects the enzyme production greatly (Hameed *et al.*, 1999).

Different inoculum sizes represented graphically (Figure 13) were investigated for their effect on productivity of the protease by B13.

The results indicated that the use of 2.0 ml of 48 h old inoculum (optical density 0.75 at 660 nm), gave the highest yield. Similar result was also found for *Streptomyces pulvereceus* MTCC 8374 by Jayasree *et al.*, (2009). Our results are similar with organism *Thermoactinomyces sp.* RM4 reported by Amit Verma *et al.*, 2016 It is well documented that an inoculum size of 2% to 5% is optimum for protease production (Mabrouk *et al.*, 1999; Kanekar *et al.*, 2002).Moreover, in the reports of Sinha and Satyanarayana (1991) and according to Gajju *et al.*, (1996) range of 1% to 8% inoculums was the optimum.

### **Detergent compatibility test**

Our crude protease (117.5 units/ml) exhibited significant compatibility with commercial laundry detergents like Wheel, Rin and Tide. The enzyme retained 99.46% of its original activity after incubating the enzyme at 37 C for 30 min before the enzyme assay in the presence of wheel and 87.23% activity in presence of Ariel.

Protease exhibited stability in the following order wheel > Tide > Rin > Ariel. Alkaline protease of B13 had shown high compatibility with most of the commercially available detergents like wheel, Tide and Rin hence, can be used efficiently as detergent additive (Figure 14).

Protease from *Bacillus* strain B13 was also reported for removal of chocolate stain and compatibility with commercial detergent. The basic requirements for proteases for their detergent application include (Gupta and Beg, 2002):

- i. Availability at low cost from safe microbial source
- ii. Capable of working in high alkaline pH of common detergent solution
- iii. Sufficiently thermostable with higher temperature optimum
- iv. Low or no allergic response for topical use
- v. Compatibility with detergent component

**Table.1** Cultural and morphological characters of keratinolytic protease producing isolates

| Isolates | Sample source   | Colony characters  | Gram reaction                                  |
|----------|---|--|--|
| A1       | Rock sample, Dalhousie, Himachal Pradesh                | Small, round, even, slightly raised, opaque, rough, white, grey pigment on aging | Gram +ve spore forming filamentous             |
| B13      | Soil sample of Manikarnam near Manali, Himachal Pradesh | Small, round, uneven, raised, smooth, opaque with red pigment after 48h          | Gram+ve rod single and in pairs, spore forming |
| M9       | Soil sample of Manikarnam near Manali, Himachal Pradesh | Small, round, uneven, slightly raised, rough, opaque, light white                | Gram+ve filamentous                            |

**Table.2** Cultural and morphological characteristics of *Bacillus* sp B13

| Isolate | Size  | Shape | Margin | Texture | Elevation       | Opacity | Colony Color | Morphology by Gram staining   |
|---------|-------|-------|--------|---------|-----------------|---------|--------------|---|
| B13     | Small | Round | Uneven | Smooth  | Slightly raised | Opaque  | Red          | Gram positive, rod shaped, arranged in pairs and in clusters with spore formation |

**Table.3** Biochemical Activity of isolate B13

| No. | Test\ Org.  | B13  |
|-----|---|--|
| 1   | M.R. test   | Positive                                     |
| 2   | V.P. test   | Negative                                     |
| 3   | Nitrate reduction test  | Positive                                     |
| 4   | Gelatin hydrolysis test   | Positive                                     |
| 5   | Catalase test   | Negative                                     |
| 6   | Indole production test  | Negative                                     |
| 7   | Growth of 6.5% NaCl   | Positive                                     |
| 8   | H <sub>2</sub> S production test  | Negative                                     |
| 9   | Citrate utilization test  | Positive                                     |
| 10  | Urea utilization (Urease activity)  | Negative                                     |
| 11  | Carbohydrate utilization test<br>1) Glucose<br>2) Arabinose<br>3) Xylose<br>4) Mannitol | Negative<br>Positive<br>Positive<br>Positive |
| 12  | N.broth (Growth pattern)  | uniform growth                               |
| 13  | Amylase Production test/ starch hydrolysis  | Positive                                     |

Fig.1 Skim milk agar medium showing colonies of protease producers

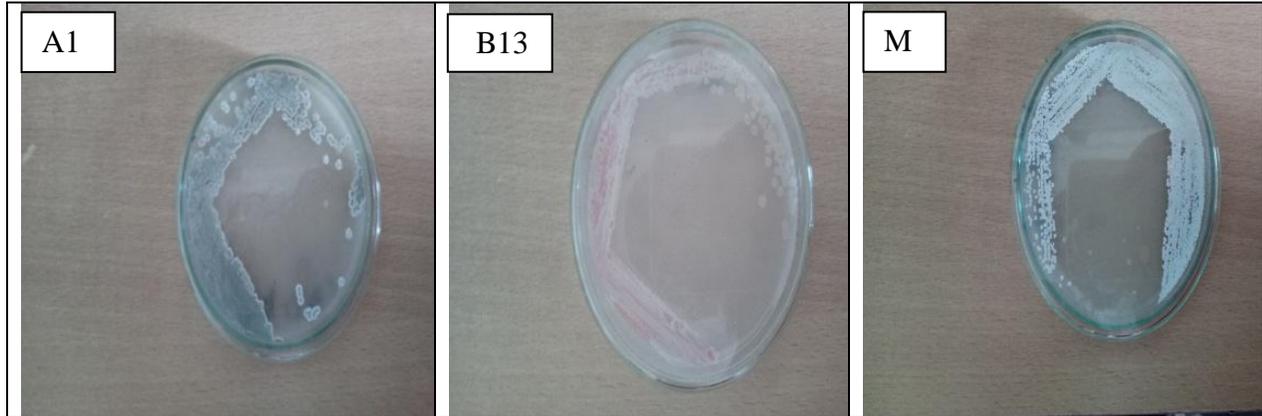


Fig.2 Relative activities of isolates

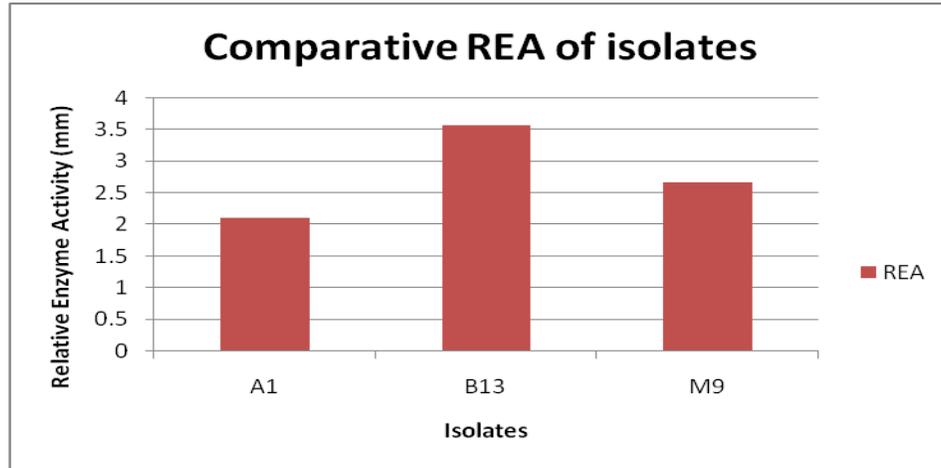
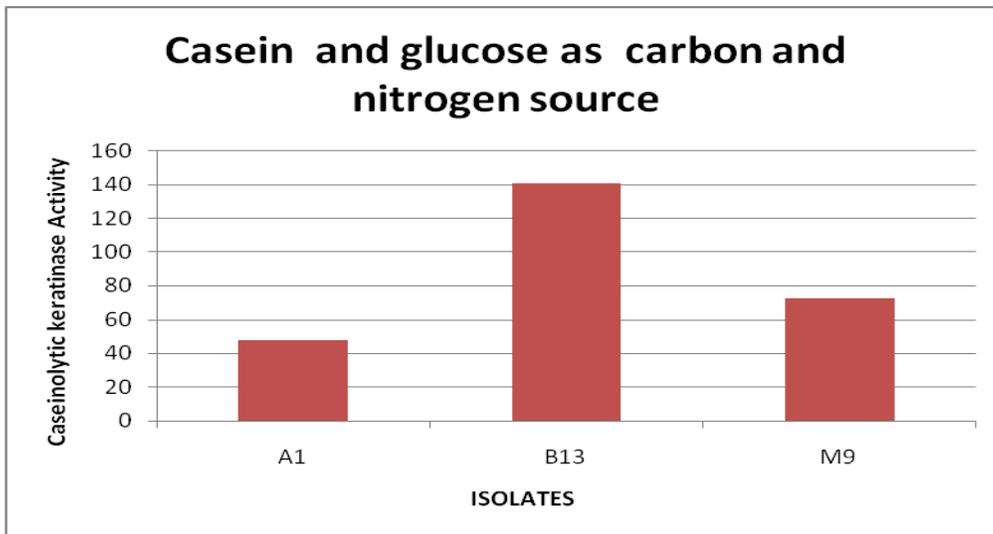
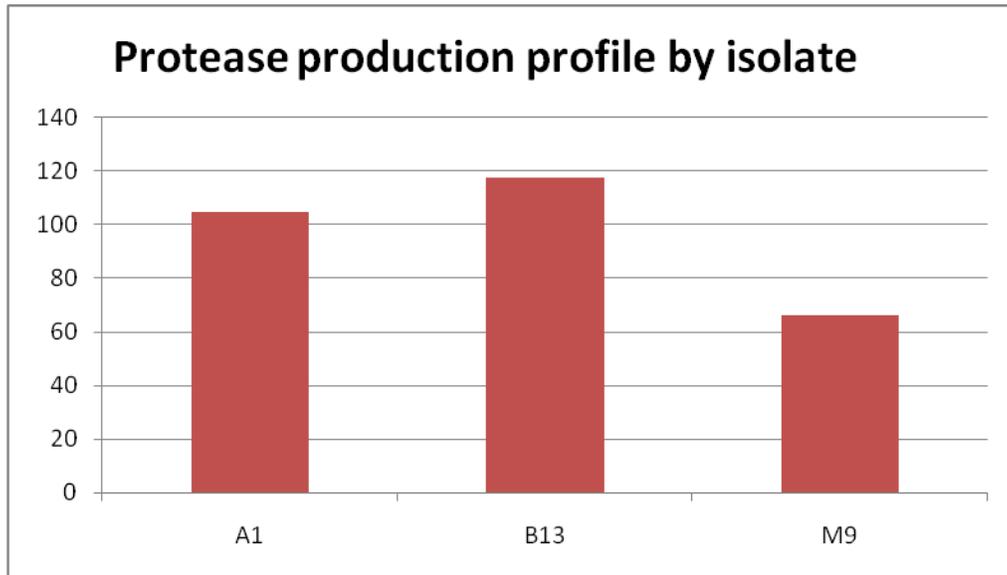


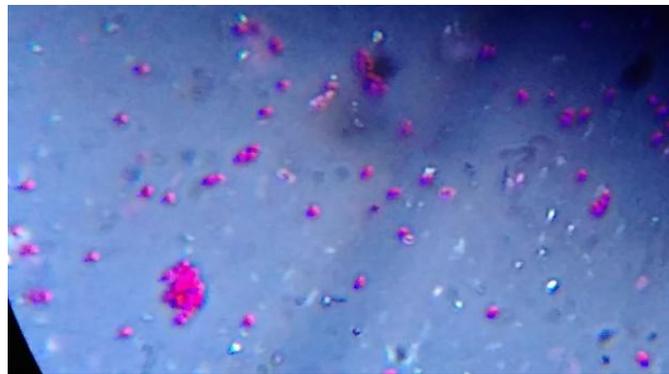
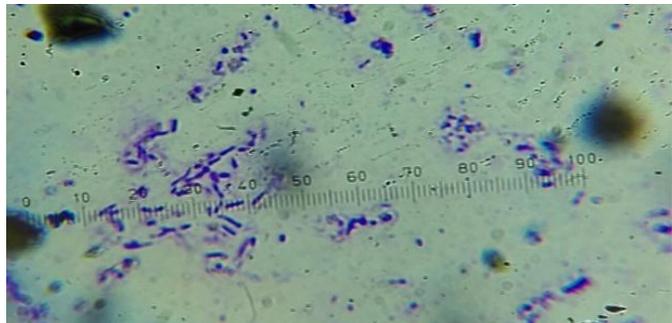
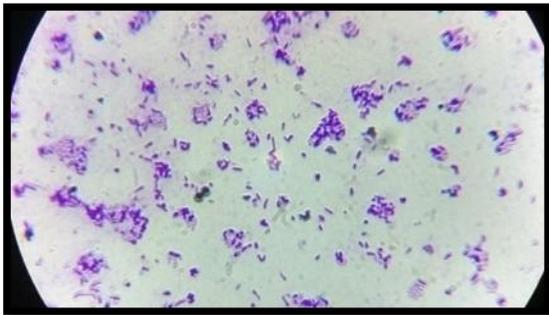
Fig.3 Alkaline protease production profile by isolates using medium suggested by Rao and Narasu (Glucose and casein as substrate)



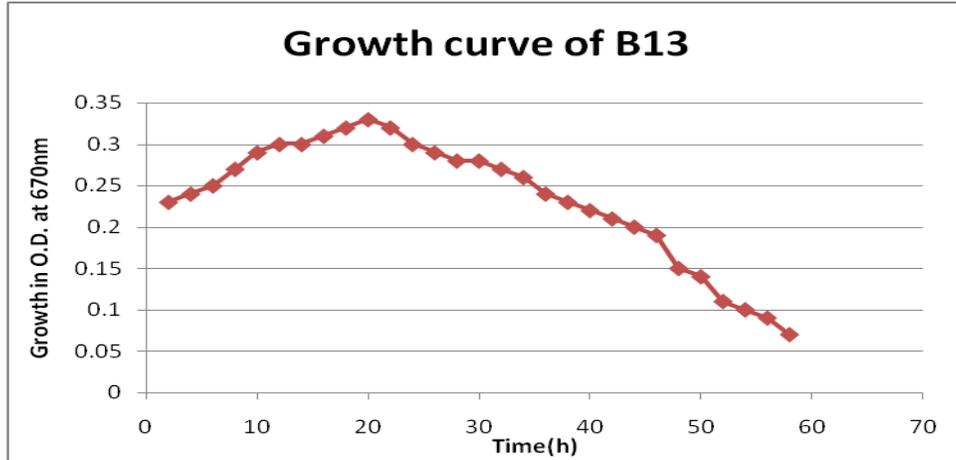
**Fig.4** Alkaline protease production by isolates using feathers as sole source of carbon and nitrogen



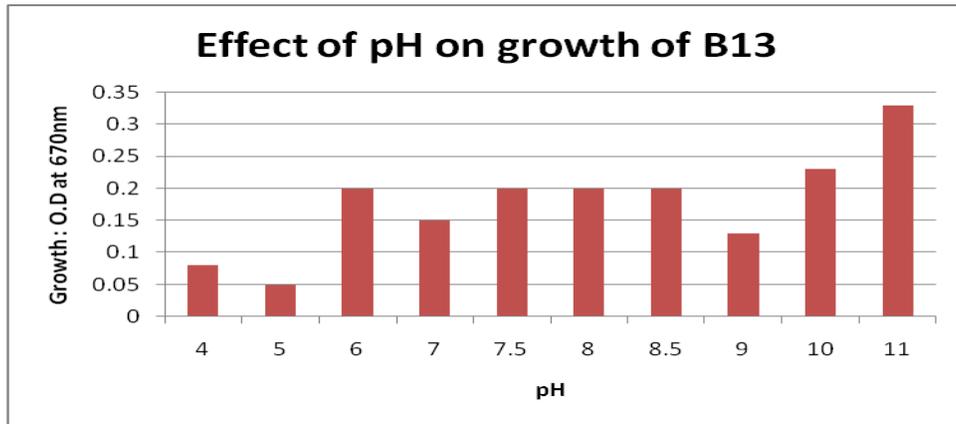
**Fig.5a.** Morphology by Gram's staining of isolate B13; b. Micrometry for measuring size of isolate B13. Length (4 $\mu$ m) and width(1 $\mu$ m); c. Spore staining performed by Dorner's method. Showing centrally located endospores



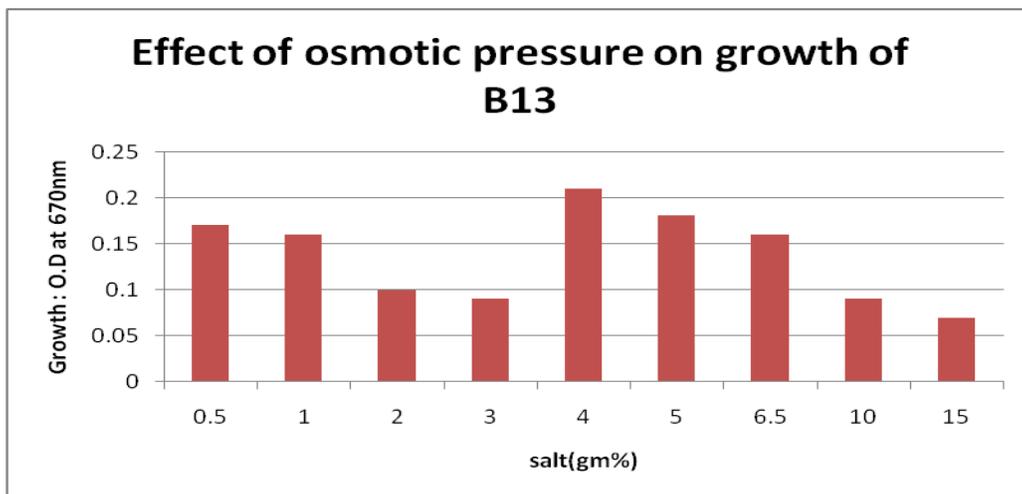
**Fig.6** Growth curve of isolate B13



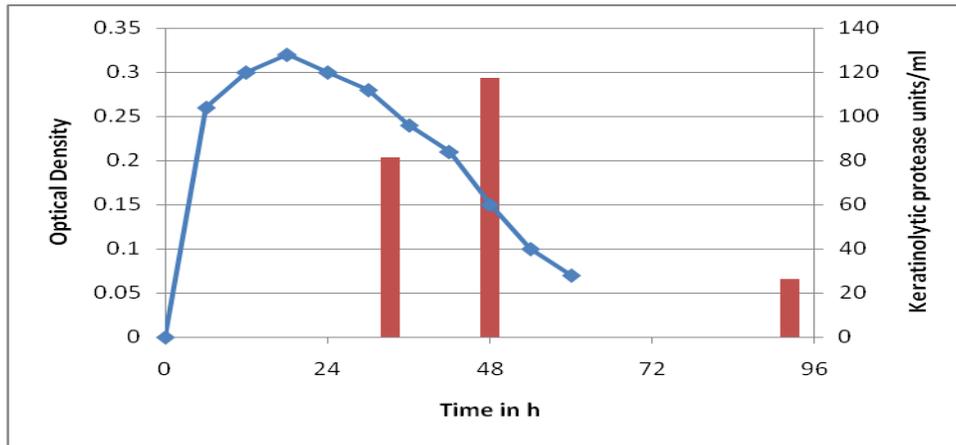
**Fig.7** pH on growth by isolate B13 in nutritive broth



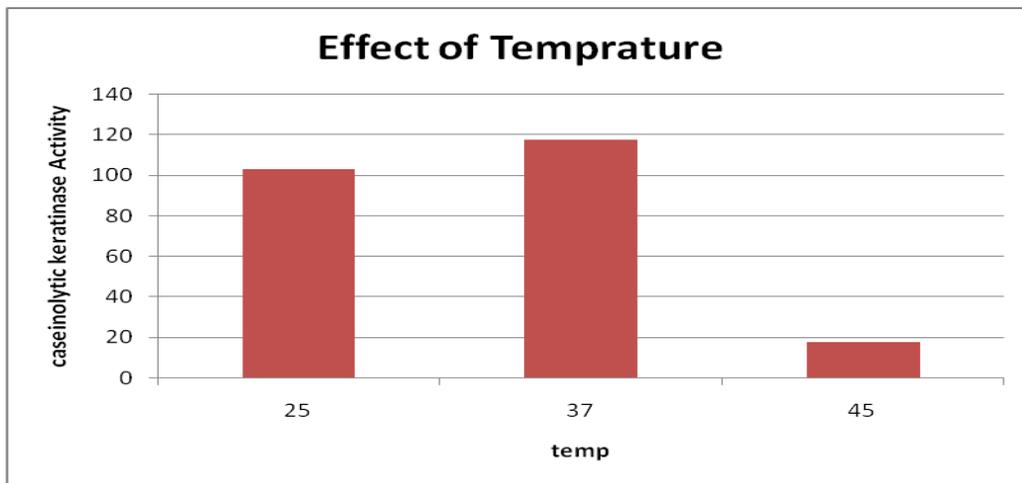
**Fig.8** Effect of osmotic pressure on growth of isolate B13



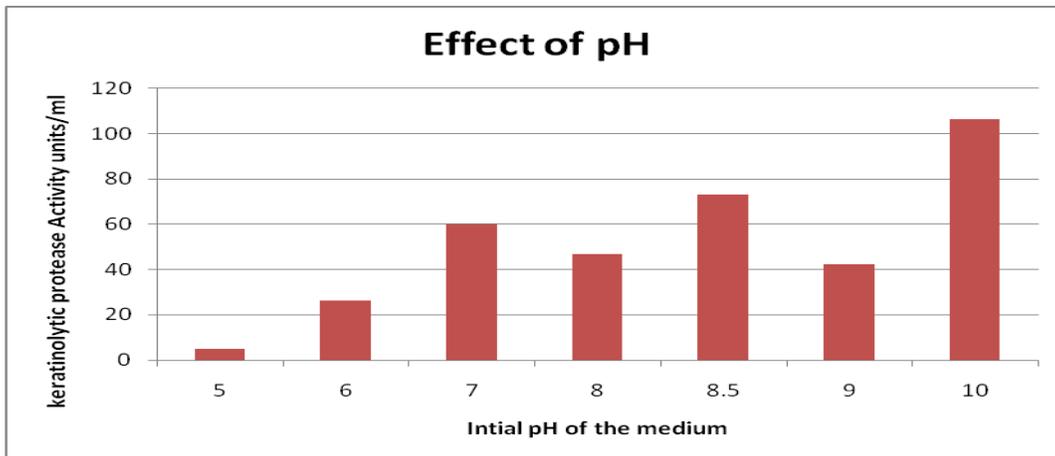
**Fig.9** The protease production profile of B13 to determine incubation period with reference to growth



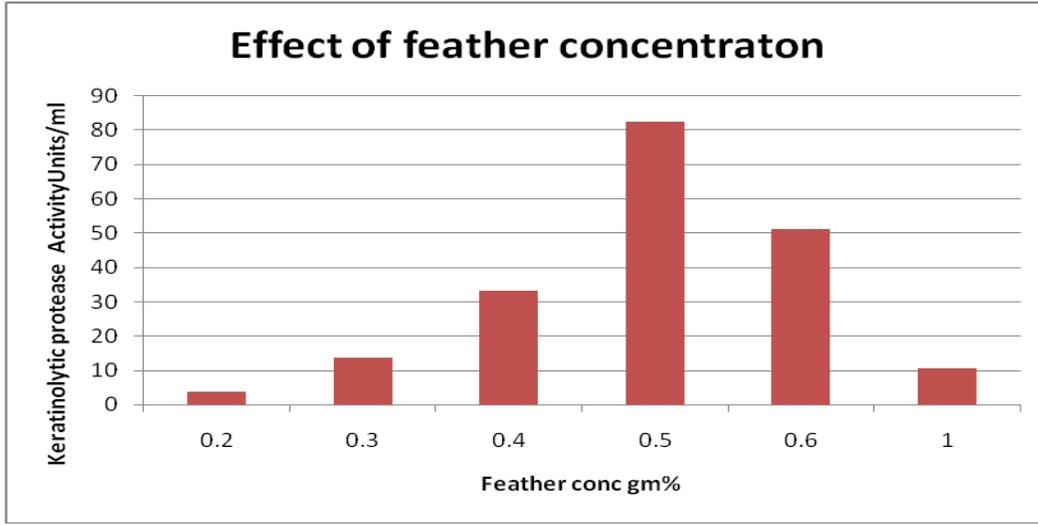
**Fig.10** Effect of temperature on protease production by isolate B13



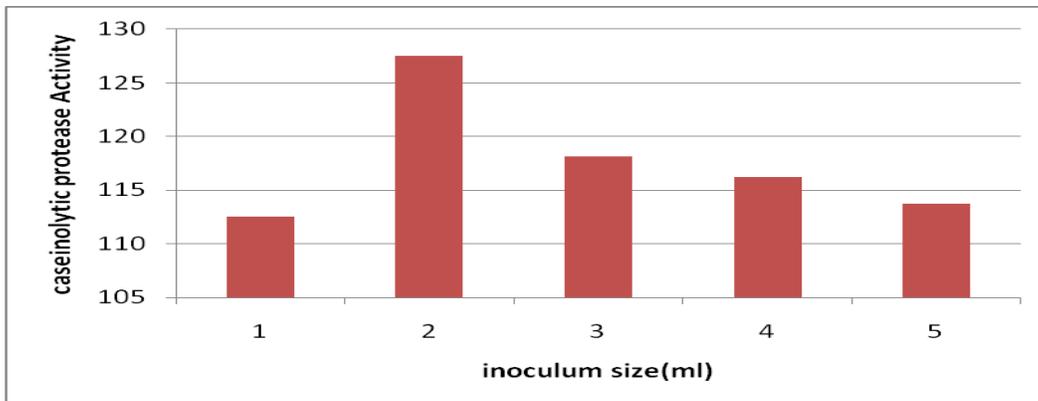
**Fig.11** Effect of medium pH on protease production



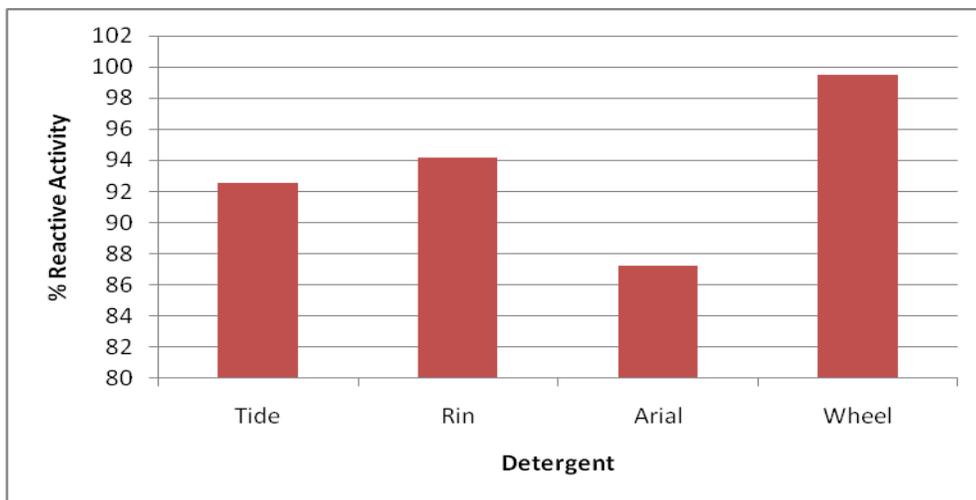
**Fig.12** Effect of feather concentration on protease production by B13.



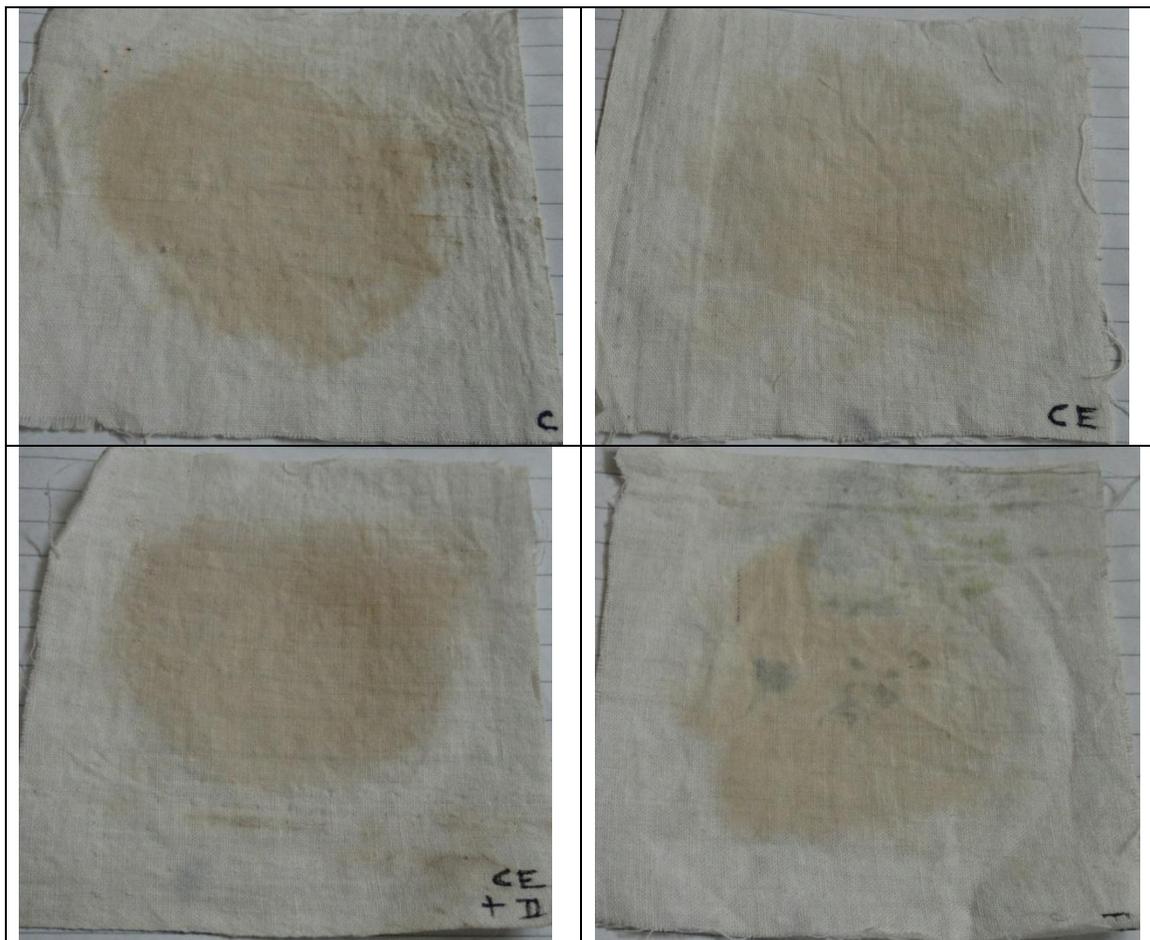
**Fig.13** Effect of inoculum volume on protease production by B13



**Fig.14** Compatibility of protease of B13 with commercial detergents



**Fig.15** Washing performance of protease of A1 (A: only water wash, B: only enzyme, C: detergent and enzyme, D:only detergent)



**Washing performance of protease of B13 was studied on white cotton cloth pieces (10×10) stained with chocolate**

After 30 min of incubation at room temperature the detergent solution supplemented with the enzyme was able to remove the chocolate stains completely, while the detergent solution only could not remove it. The best washing performance was recorded by washing with only enzyme, followed by washing with detergent, washing with enzyme+detergent and washing with only water. Bhosale *et al.*, (1995) have reported high activity of alkaline protease of *Conidiobolus coronatus* showing compatibility at 37 C in the presence of 25 mM CaCl<sub>2</sub> with varieties of detergents.

Adinarayana *et al.*, (2003) worked with *Bacillus subtilis* PE II proteases and reported its compatibility and stability with various locally available detergents at 37 C. same kinds of studies were also reported for proteases from *Siplosoma obliqua* (Anwar and Saleemuddin, 1997), *Bacillus brevis* (Banerjee *et al.*, 1999), *Bacillus cereus* (Kanmani *et al.*, 2011).

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